J. Membrane Biol. 22, 53–72 (1975)
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A Comparison of the Rate Equations, Kinetic Parameters, and Activation Energies for the Initial Uptake of L-Lysine, L-Valine, γ-Aminobutyric Acid, and α-Aminoisobutyric Acid by Mouse Brain Slices*

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Received 20 May 1974; revised 29 January 1975

Summary. At substrate concentrations, in medium, of 0.2 to 20 mM and at temperatures of 25 and 37 °C, the initial concentrative influx of the amino acids L-lysine (30 and 37 °C), L-valine, and γ-aminobutyric acid into incubated mouse-cerebrum slices follows the rate equation for the initial influx of α-aminoisobutyric acid (Cohen, *J. Physiol.* **228**:105, 1973), $v = V_{max}/(1 + K_t/S) + k_u S$. Kinetic constants at 37 °C are: $V_{max} = 0.089$ μmoles/g final wet wt of slices, min, $K_t = 0.69$ mM, $k_u = 0.037$ μmoles/g final wet wt, mM-substrate, min for L-lysine; $V_{max} = 0.60$, $K_t = 1.30$, $k_u = 0.067$ for L-valine; and $V_{max} = 1.71$, $K_t = 1.58$, $k_u = 0.094$ for γ-aminobutyric acid. The linear term, $k_u S$, is due to an unsaturable process of concentrative uptake, not diffusion. Comparison of temperature coefficients reveals a "reference" pattern for typical low affinity transport of amino acids into brain slices. Its characteristics are: Activation energies associated with V_{max} and k_u are in range 14 to 20 kcal/mole; K_t varies only slightly with temperature. L-Lysine and α-aminoisobutyric acid fit this pattern; L-valine and γ-aminobutyric acid deviate in part. The Akedo-Christensen plot (*J. Biol. Chem.* **237**:118, 1962) does not distinguish between the rate equation $v = V_{max}/(1 + K_t/S) + k_u S$ for saturable uptake plus first-order unsaturable concentrative uptake, and the rate equation $v = V_{max}/(1 + K_t/S) + k_D(S - S_i)$ for saturable uptake plus first-order non-concentrative "passive diffusion".

The transport of amino acids to and from central nervous tissue has been studied extensively for a number of years. (*See* Neame, 1961; Cohen & Lajtha, 1972 for reviews.) Little is known about the detailed rate equations and activation energies for these processes *in vitro*, and some of the published studies are questionable. Recently, the author (Cohen, 1973*a*) found that the influx of the nonmetabolizable amino acid analog, α-aminoisobutyric

* Presented in part at the Fifth Meeting of the American Society for Neurochemistry, New Orleans, March, 1974 (*Trans. Amer. Soc. Neurochem.* **5**:92, 1974).

acid [1], follows a rate equation (Eq. 1) that indicates two parallel components, one saturable and one unsaturable, and he measured the three parameters, V_{max}, K_t, and k_u, in the rate equation and their temperature coefficients. This paper presents a continuation of those studies which was specifically undertaken to answer the following questions: 1) To what extent are the results valid for natural, metabolizable L-α-amino acids? and 2) How do the rate equation and temperature coefficients for the uptake of the putative neurotransmitter, GABA, compare with those for nonneurotransmitter α-amino acids despite the differences in structure and function, and despite the evidence that they are transported by different carriers (Cohen & Lajtha, 1972)? Since AIB is believed to be carried by the transport system for small neutral amino acids (Cohen & Lajtha, 1972), the α-amino acids L-lysine (large basic amino acids), and L-valine (large neutral amino acids) were chosen for this study. To allow valid comparisons with the other amino acids the low-affinity transport of GABA was studied.

Materials and Methods

The initial rate of influx of the three amino acids was measured following the procedure used to study the kinetics of AIB influx (Cohen, 1973*a*). Six- to nine-week old Swiss mice from the Institute colony were decapitated and the brain rapidly removed. The olfactory bulbs and underlying white matter were trimmed from the cerebral hemispheres, which were then immersed in chilled medium for a few seconds. The tissue was removed from the medium, blotted, and nominally 0.37-mm thick slices cut with the calibrated (Cohen, 1974) McIlwain-Buddle (1953) tissue chopper as described by Blasberg and Lajtha (1965). (Males were used almost exclusively even though no difference between the sexes was observed in the earlier study.) Slices from one hemisphere (about 125 to 150 mg) were placed in 4.5 ml of oxygenated, substrate-free medium at temperature in a 25-ml stoppered erlenmeyer flask, and preincubated for 30 min in a thermostatted, reciprocating water bath. One-half ml of medium at temperature containing ^{14}C-labeled substrate with carrier at 10 times the desired concentration was quickly added and incubation continued for a predetermined interval. The tissue was rapidly filtered off with suction. The resulting tissue pellet was frozen in dry ice, weighed, homogenized in 5 per cent (w/w) perchloric acid, and the concentration of substrate (actually acid-soluble ^{14}C-labeled substances) determined by liquid scintillation counting as described previously (Cohen, Blasberg, Levi & Lajtha, 1968; Cohen, Stampleman & Lajtha, 1970). The range of incubation periods in which uptake increased linearly with time (Fig. 1) was determined for 0.2 and 20, or 0.2, 2 and 20 mM substrate. The rate of net influx was calculated from the slope of this linear region. At intermediate concentrations, tissue was incubated with substrate for either of two periods within the linear region (e.g. 6 and 14 min for L-lysine at 30 °C); the rate of net influx during this period was calculated. These procedures were adopted to eliminate the contribution of influx

1 *Abbreviations:* AIB, α-aminoisobutyric acid; GABA, γ-aminobutyric acid; HEPES, N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid.

into the *functional* extracellular space for amino acid uptake (Cohen, 1972) since this tissue compartment cannot be measured at present.

To determine the fraction of ^{14}C-label in metabolic products, slices were incubated with typical concentrations of each substrate, then filtered off and frozen as described above. The frozen pellet was weighed, homogenized in 3 per cent sulfosalicylic acid, and centrifuged. Clear supernatant was placed on a column of Aminex A-6 resin (Biorad Laboratories, Richmond, Calif.) for amino acid analysis, and eluted with lithium citrate buffer as described by Perry, Stedman and Hansen (1968). The stream from the column was split; one portion went to a fraction collector to get samples for liquid scintillation counting; the other was continuously mixed with ninhydrin reagent, and then passed through a recording colorimeter to measure separated peaks.

Tissue was incubated in standard HEPES-buffered medium (Cohen *et al.*, 1970). This contained (in mM): NaCl 119; KCl 5.0; $CaCl_2$ 0.75; $MgSO_4$ 1.2; NaH_2PO_4 1.0; $NaHCO_3$ 1.0; HEPES 25; NaOH 12, which reacts to form the complementary base of the buffer; and glucose, 10 mM. It has a nominal tonicity of 300 milliosmoles. The $pH = 7.35 - 0.0116\ (t - 25)$, where t is the temperature in °C. Labeled amino acids were from New England Nuclear Corp. They gave single spots with ascending paper chromatography using butanol-acetic acid-water (60:15:25 by volume) and methanol-pyridine-water (80:4:20 by volume) as solvents. Other chemicals were reagent grade or the best available from various sources.

When the rate of influx was to be calculated from the slope of a plot of the concentration of substrate in tissue as a function of time, each data point was determined in triplicate, and a straight line was fitted by least squares on y to at least four sets of data points; when the rate was to be calculated from the concentration in tissue at two incubation times, six replicates were made. The kinetic parameters in Table 1 were computed by an iterative procedure in which the sum of the squares of the relative errors (observed rate − calculated rate)/(calculated rate), was minimized. The relative standard deviation of data points from the best fit ranged from 5 per cent with GABA to 11 per cent with L-valine. Because the three parameters, V_{max}, K_t, and k_u are not linearly independent, and because Barber, Welch and Mackay's (1967) assumption about the nature of errors in V_{max} and K_t is not applicable, the precision of these parameters was not estimated. The precision should be roughly the same as in the study of AIB uptake (Cohen, 1973*a*), that is: V_{max}, ±10%; K_t, ±5%; and k_u, ±10%. E_a and ΔH were calculated from Eq. (3). Only two temperatures were used because in studies of the kinetics of AIB uptake (Cohen, 1973*a*) it was found that within experimental precision ln(parameter) varied linearly with 1/(absolute temperature) as required by this equation.

Rates were computed in units of μmoles/g final wet weight of tissue × min instead of μmoles/g intracellular water × min, because the functional extracellular space is not known. This does not affect K_t, $(E_a)_{V_{max}}$, $(E_a)_{k_u}$, and $(\Delta H)_{K_t}$; it decreases V_{max} and k_u by the factor (g intracellular water)/(g final wet weight of tissue).

Results

Evidence for Concentrative Uptake

The concentration of ^{14}C in tissue (expressed as equivalent relative concentration of substrate assuming no metabolism) is shown in Fig. 1 as a function of incubation time. All four curves have a linear portion where the net influx of ^{14}C is constant. This portion was taken to represent the region in which the predominant transport process is uptake of substrate by the

cells. The curves for L-lysine, and L-valine have an initial portion showing a more rapid, but monotonically decreasing influx. This portion probably represents penetration into the functional extracellular space (i.e. the extracellular space and any portions of the intracellular space that behave as extracellular space for the substrate). The curves for L-valine and GABA at 37 °C have a final portion where the rate of uptake steadily drops. Presumably here efflux, control mechanisms or both decrease the rate of uptake of substrate. For the determination of the rate of entry it is unimportant whether the measured concentration of ^{14}C is in substrate or in metabolic products, because the fate of a substance after it has entered a cell has at most only a secondary effect on the transport process. A knowledge of the concentration of the substrate within the tissue, as distinct from the equivalent concentration of the radioactive isotope used as a label, is essential to determine whether there has been concentrative uptake, and to distinguish unsaturable transport from passive diffusion. The fraction of ^{14}C-label in metabolites was small under the experimental conditions, not exceeding 10 percent for GABA, the most rapidly metabolized of the three substrates. Even with a correction for about 10 per cent of ^{14}C in metabolic products, Fig. 1 clearly shows concentrative initial uptake.

The Rate Equation

The initial rate of entry of the three amino acids, after the extracellular space has been filled, is shown as a function of concentration in Figs. 2 to 4. The values fit the rate equation for saturable plus unsaturable uptake that was previously established for the initial influx of AIB into brain slices (Cohen, 1973*a*)

$$v = V_{max}/(1 + K_t/S) + k_u S \tag{1}$$

where v is the rate of initial uptake, V_{max} is the maximum rate of the Michaelis-Menten (saturable) component, K_t is the Michaelis constant for transport, S is the substrate concentration in the medium, and k_u is the first-order rate constant for the unsaturable component. The curves were computed from the constants in Table 1.

The process represented by the first-order kinetic term in this equation must be clearly distinguished from "passive diffusion" into cells. A term for passive diffusion would have the form, $k_D(S - S_i) = k_D S(1 - S_i/S)$, where k_D is the rate constant for diffusion, and S_i is the intracellular concentration of substrate; in terms of S_t, the measured concentration of substrate in tissue, the diffusion term would be $(k_D/w_i) S(w - S_t/S)$, where w_i

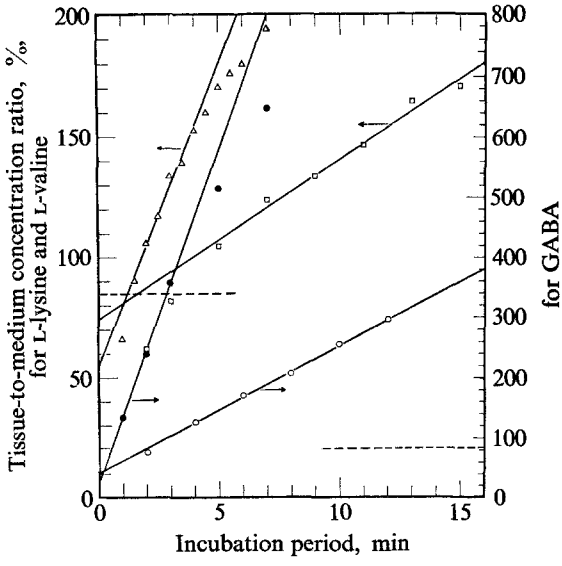

Fig. 1. Uptake of amino acids by brain slices. •, 0.2 mM GABA at 37 °C; ○, 2 mM GABA at 25 °C; □, 0.2 mM L-lysine at 30 °C; △, 2 mM L-valine at 37 °C. The interrupted lines at 84 per cent indicate the tissue-to-medium concentration ratio where the concentration of substrate in tissue water is equal to its concentration in the medium. Read uptake of L-lysine and L-valine on the left-hand scale; uptake of GABA on the right-hand scale

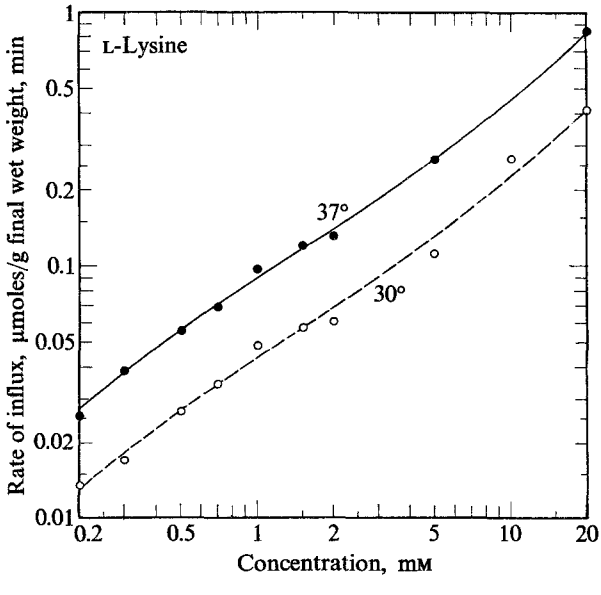

Fig. 2. The rate of influx of L-lysine into brain slices as a function of its concentration in the medium

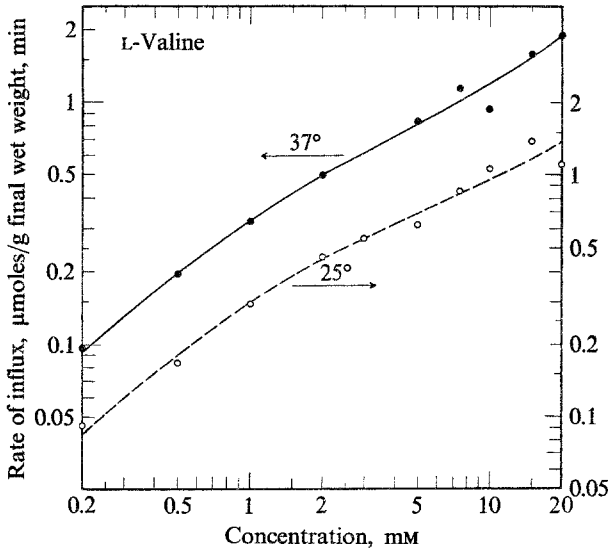

Fig. 3. The rate of influx of L-valine into brain slices as a function of its concentration in the medium. Read influx at 37 °C on the left-hand scale; influx at 25 °C on the right-hand scale

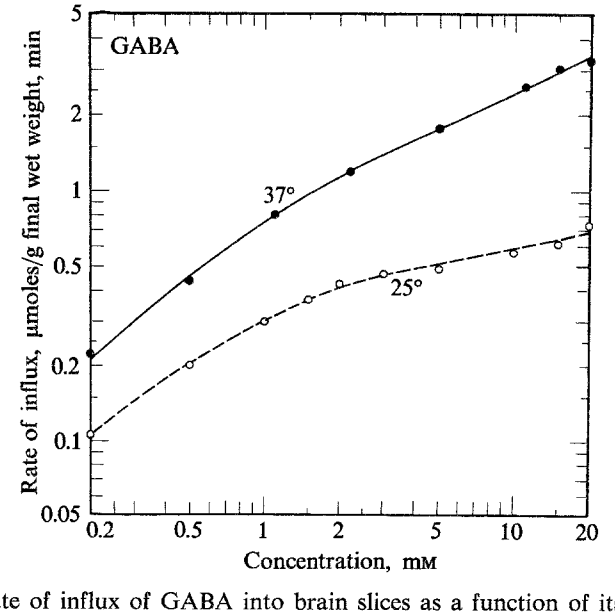

Fig. 4. The rate of influx of GABA into brain slices as a function of its concentration in the medium

is the fraction of tissue that is intracellular water, and w is the total fraction of tissue that is water. The rate of uptake by passive diffusion decreases as the concentration of substrate in tissue water increases, and becomes negative

Table 1. Kinetic parameters and corresponding activation energies or standard enthalpies for the uptake of amino acids by mouse brain slices

Substrate	Temperature (°C)	V_{max} (μmoles substrate/g final wet wt × min)	K_t (mM)	k_u (μmoles substrate/g final wet wt mM-substrate × min)
L-lysine	37	0.089	0.69	0.037
	30	0.042	0.71	0.019
$(E_a)_{V_{max}}$, $(\Delta H)_{K_t}$, $(E_a)_{k_u}$ (kcal/mole)		20	−0.6	18
L-valine	37	0.60	1.30	0.067
	25	0.66	1.51	0.039
$(E_a)_{V_{max}}$, $(\Delta H)_{K_t}$, $(E_a)_{k_u}$ (kcal/mole)		−1.3	−2.2	8.2
GABA	37	1.71	1.58	0.094
	25	0.58	0.92	0.0066
$(E_a)_{V_{max}}$, $(\Delta H)_{K_t}$, $(E_a)_{k_u}$ (kcal/mole)		17	8.2	41
AIB [a]	37	0.39	1.12	0.054
	25	0.16	1.06	0.017
$(E_a)_{V_{max}}$, $(\Delta H)_{K_t}$, $(E_a)_{k_u}$ (kcal/mole)		14	0.7	17

There are some slight discrepancies between kinetic parameters and corresponding energies because energies were computed from unrounded values. Kinetic parameters and energies were then rounded off independently.

[a] Values for AIB are from Cohen (1973*a*).

(efflux) when the concentration in tissue water exceeds the concentration in the medium. The tissue water in these slices is about 84 per cent of final wet weight (Cohen *et al.*, 1970), and therefore if this process were diffusive, k_u would have been negative when S_t/S was greater than 0.84. The rate constants, k_u, (Table 1) are positive even though, as Fig. 1 shows, they were computed from rates measured with tissue concentrations greater than medium concentrations. Consequently, this process cannot be "passive diffusion", but must be true concentrative uptake.

Kinetic Parameters and Their Associated Energies

The kinetic parameters, Arrhenius activation energies for V_{max} and k_u, and the van't Hoff standard enthalpy (heat of reaction) for K_t are listed in Table 1. The values for GABA are for the so-called "low affinity" uptake

Table 2. Kinetic parameters from the literature for the uptake

Preparation	Medium	Temperature (°C)	Amino acid
Rat cerebral cortex prisms, 0.26 × 0.26 mm	Oxygenated Krebs-Ringer's phosphate (pH 7.4) with glucose	37	AIB L-alanine D-alanine glycine
Adult rat cerebral hemisphere, 0.5-mm 1st slice	Oxygenated Krebs-Ringer's phosphate (pH 7.4) with glucose	37	L-phenylalanine L-tyrosine L-tryptophan L-histidine L-leucine
Adult rat cerebral hemisphere, 0.35-mm slices	Oxygenated Krebs-Ringer's bicarbonate with glucose	35	glycine L-glutamate
Rat hypothalamus median eminence	Oxygenated Krebs-Ringer's bicarbonate (pH 7.4) with glucose	37	AIB

Notes:

[a] V_{max} and k_u were reported per mg protein. They have been recalculated assuming tissue contained 18 per cent dry weight which was 40 per cent protein.

[b] Rates were calculated from substrate in tissue after 5-min incubation minus substrate in tissue after 0-min incubation.

[c] $k_u(k_D)$ was referred to as a diffusion constant but was calculated as though it represented unsaturable first-order uptake.

[d] V_{max} and k_u were reported per gram final wet weight of tissue.

[e] Substrate in tissue was corrected for amount in "extracellular space" = inulin space.

[f] V_{max} and k_u were reported per ml intracellular water; they have been recalculated assuming tissue initially had 20 per cent dry weight and an extracellular space of 20 per cent by weight.

which is predominant with substrate concentrations of about 0.1 mM or greater. From these tabulated rate constants the order of the rate of initial uptake throughout the concentration range employed (0.2 to 20 mM) is GABA > L-valine (large neutral amino acid) > AIB (small neutral amino acid) > L-lysine (large basic amino acid), which differs from the commonly accepted order for brain *in vitro* – GABA > small neutral amino acids > large neutral amino acids > large basic amino acids (Cohen & Lajtha, 1972) – only by the inversion of the order of small and large neutral amino acids. The relative order of amino acid concentrations after long-term incubations or at "steady (?) state" has sometimes been considered to parallel the relative

of amino acids by preparations of central nervous tissue

V_{max} (μmoles substrate/g final wet wt × min)	K_t (mM)	k_u (μmoles substrate/g final wet wt mM-substrate × min)	Notes	Ref.
0.177	0.43	0.120	a, b, c	1
0.304	0.44	0.157		
0.318	1.47	0.131		
0.429	0.44	0.173		
0.101	0.66	0.028	c, d, e	2
0.101	0.47	0.030		
0.038	0.30	0.032		
0.145	1.18	0.026		
0.062	0.40	0.026		
0.078	5.4	0.041	e, f, g	3
0.39	2.22	0.037		
0.063	0.77	0.025	g, h	4

[g] The Akedo-Christensen (1962) plot was used to evaluate kinetic parameters. V_{max} and k_u have been recalculated assuming unsaturable first-order kinetics.

[h] V_{max} and k_u were reported per ml intracellular water; they have been recalculated using the author's values for incubated tissue of 2.80 ml/g dry weight extracellular water, and 4.35 ml/g dry weight intracellular water. The authors used "extracellular space" = polyethylene glycol space.

References: 1. Smith (1967). 2. Vahvelainen and Oja (1972). 3. Laššánová and Brechtlová (1971). 4. Silverman, Knigge and Peck (1972).

rate of initial uptake. There need not be a close correlation, because long-term tissue concentrations depend on efflux and metabolism as well as on influx.

Table 2 contains some of the more reliable literature values of V_{max}, K_t, and k_u for preparations of central nervous tissue. For the most part they agree moderately well with the values in Table 1. There are no serious discrepancies in the values for V_{max}. The values for K_t are somewhat lower, but with few exceptions are consistent with an approximate value of 1 mM for the low affinity uptake systems in brain (Cohen & Lajtha, 1972). Laššánová and Brechtlová's (1971) values appear high; their value for glycine may be wrong. Smith's (1967) values are appreciably higher than other values for k_u in Tables 2 and 3.

Table 3. Contribution of unsaturable system to influx, in per cent of total uptake

Preparation	Temperature (°C)	Amino acid	Concentration of substrate in medium (mM)				
			0	0.2	2	20	Ref.
Mouse cerebrum slices	37	L-lysine	23	27	58	90	1
	30		24	29	55	86	
	37	L-valine	13	14	27	70	
	25		8.3	9.2	17	56	
	37	GABA	8.0	8.9	16	54	
	25		1.1	1.3	3.3	19	
Mouse cerebrum slices	37	AIB	13	16	30	75	2
Rat cerebrum slices	37	glycine	15	20	50	89	3
	37	AIB	22	30	62	93	
Rat cerebral cortex slice	37	L-phenylalanine	15	19	42	86	4
	37	L-leucine	14	20	50	90	
Rat cerebrum slices	35	glycine	22	23	28	57	5
Rat hypothalamus median eminence slices	37	AIB	3.4	4.2	11	48	6
Rabbit choroid plexus	37	L-proline	3.8	4.4	9.5	41	7
Rabbit iris ciliary body	37	L-proline	29	31	41	77	8
Embryonic chick heart	37.5	AIB	3.0	3.2	5.4	23	9
Ehrlich ascites cells	37	L-tryptophan	4.1	4.8	11	45	10
	37	AIB	0.30	0.33	0.61	3.4	

References: 1. Present work. 2. Cohen (1973*a*). 3. Smith (1967). 4. Vahvelainen and Oja (1972). 5. Laššánová and Brechtlová (1971). 6. Silverman, Knigge, and Peck (1972). 7. Coben *et al.*, (1971). 8. Coben *et al.* (1970). 9. Guidotti *et al.* (1968). 10. Jacquez, Sherman, and Terris (1970).

Regretably, the literature contains few acceptable studies of amino acid transport to which the effects of temperature presented in Table 2 may be compared. The most reliable one was made on Ehrlich ascites cells by Jacquez, Sherman and Terris (1970). (The authors' assumption that k_u is a diffusion parameter did not affect their calculated activation energies.) The activation energies for V_{max} [their $J_M(3)$] and for k_u (their k_3) agree more or less with the "reference" pattern shown by the uptake of AIB and L-lysine by brain slices (*see* Discussion). Activation energies for V_{max} are: L-methionine, 11.1; L-tryptophan, 13.0; glycine, 15.5; L-alanine, 13.0; AIB, 16.3; and L-phenylalanine, 17.5 kcal/mole; those for k_u are: L-methionine, 17.3; L-tryptophan, 11.8; glycine, 17.7; and L-phenylalanine, 15.9 kcal/mole. The temperature coefficient of K_t [their $K_m(3)$] depended on the amino acid. The K_t for L-methionine, glycine, and L-alanine showed little or no temperature dependence ($\Delta H = 0$ kcal/mole); the K_t for L-tryptophan ($\Delta H =$ 14.7 kcal/mole) and L-phenylalanine ($\Delta H = 20.3$ kcal/mole) increased rapidly

with temperature; and the K_t for AIB decreased with rising temperature ($\Delta H = -5$ to -7 kcal/mole, estimated from the published van't Hoff graph).

Discussion

Comparisons Among the Substrates

A well-established phenomenological rate equation is a most useful guide in the study of transport mechanisms since any postulated mechanism must conform to it. In this and the previous study (Cohen, 1973*a*), the rate equation shows the existence of two parallel pathways for concentrative uptake; the rate equation from studies of the efflux of exogenous amino acids from brain slices shows the presence of three kinetically distinct tissue compartments, and indicates that there may be two parallel pathways for efflux from one of them (Cohen, 1973*b*). The mechanisms of transport processes with different rate equations obviously must differ at least in part. However, even when several transport processes follow the same rate equation, they can be qualitatively different; such differences may be indicated by comparisons among corresponding parameters, and especially among their temperature coefficients.

Such comparisons reveal a provisional "reference" pattern for typical low-affinity amino acid transport systems in brain slices. Its essential features are: 1) E_a corresponding to V_{max} is in the range 14 to 20 kcal/mole. This is within the range for enzyme-catalyzed reactions (Dixon & Webb, 1964) and ordinary chemical reactions proceeding at reasonable rates at room temperature. 2) E_a corresponding to k_u is in the same range, 14 to 20 kcal/mole. 3) K_t varies only slightly with temperature; that is Q_{10} is in the range 0.85 to 1.15 at ordinary temperatures, or ΔH is approximately -2.5 to $+2.5$ kcal/mole. A fourth characteristic, K_t more or less equal to 1 mM which is common to all four, is taken as the distinguishing criterion of low affinity transport. The magnitudes of the rate constants of the saturable and unsaturable components were not considered, because until the density of transport sites is known and until the interaction, if any, of these two components is known, any limits would be unduly restrictive.

L-Lysine and AIB fit this pattern completely. The other two deviate markedly in certain respects. GABA has an appreciable positive temperature coefficient and an appreciable positive ΔH for K_t, and a value for the E_a corresponding to k_u which, on its face value, 41 kcal/mole, is much too high for typical enzyme-catalyzed reactions or for any normal chemical reaction proceeding at a moderate rate at room temperature. L-Valine has low activation energies for both rate constants.

The energies E_a and ΔH are theoretical quantities defined by

$$Q = -R\,\partial \ln P/\partial(1/T) \tag{2}$$

where P is a rate constant or an equilibrium constant (V_{max}, k_u, K_t, etc.), Q is the corresponding energy (E_a, or ΔH), R is the gas constant, and T is the absolute temperature. For convenience, an average value for Q over a temperature range is often calculated from the integrated form

$$Q = -R \ln(P_2/P_1)/(1/T_2 - 1/T_1) \tag{3}$$

the assumption being made that Q varies only slowly with temperature. Even when the quantities denoted by P are well defined, distinct phenomenological parameters, as in the present study, the interpretation of E_a or ΔH may not be straightforward. For a transport process (or a reaction) following Michaelis-Menten kinetics, $V_{max} = k\sigma$, where k is a first-order rate constant, and σ is the density of transport sites (or active sites per unit quantity of enzyme preparation), and $(E_a)_{V_{max}} = (E_a)_k - R\,\partial \ln \sigma/\partial(1/T)$. The corresponding expression for $k_u = k_u'\sigma$ is $(E_a)_{k_u} = (E_a)_{k_u'} - R\,\partial \ln \sigma/\partial(1/T)$, and for $k_u = V_{max_2}\sigma_2/K_{t_2}$ (*see below*) is $(E_a)_{k_u} = (E_a)_{V_{max_2}} - (\Delta H)_{K_{t_2}} - R\,\partial \ln \sigma_2/\partial(1/T)$. In many cases, especially for transport processes, σ is not known. Usually σ is implicitly assumed to be independent of temperature [$\partial \ln \sigma/\partial(1/T) = 0$]. However it need not be. A temperature-dependent change in the configuration of a membrane (or an enzyme) could vary the number of functional transport sites (or effective active sites of the enzyme). An increase in σ may be the simplest explanation for an unreasonably high activation energy. If σ for the unsaturable transport sites for GABA increased by a factor of 4 to 5 as the temperature increased from 25 to 37 °C, the true activation energy for this process would be reduced to 16 to 19 kcal/mole, a value consistent with the reference pattern.

In terms of rate constants, the Michaelis constant for transport is $K_t = (k_{-1} + k_2)/k_1$, where k_1 is the rate constant for the association of substrate with carrier, k_{-1} is the rate constant for the dissociation reaction, and k_2 is the rate constant for the transport of bound substrate away from the surface[2]. The dissociation reaction is often much more rapid than transport (i.e. $k_{-1} \gg k_2$). A virtual equilibrium then exists; $K_t \approx 1/K_{binding} = k_{-1}/k_1$; and $(\Delta H)_{K_t} \equiv -R\,\partial \ln K_t/\partial(1/T)$ is equal to the standard enthalpy (heat) of the dissociation reaction. If not, then $(\Delta H)_{K_t}$ no longer measures the heat

2 Rate constants are defined in terms of a carrier model for convenience only. This expression for K_t is equally appreciable to other models for the mechanism of transport that follows Michaelis-Menten kinetics.

of binding but is a complex function of the activation energies for the three rate processes, $(\Delta H)_{K_t} = [1/(k_{-1} + k_2)][k_{-1}(E_a)_{k_{-1}} + k_2(E_a)_{k_2}] - k_1(E_a)$. There are two obvious explanations for the marked temperature dependence of K_t for GABA in contrast to the other amino acids. The first is that GABA, which has a 2-carbon chain between the amino and carboxyl groups, is bound differently to its carrier than the others, which have these groups adjacent. The second is that a virtual equilibrium exists for the other amino acids, but not for GABA; that is, k_2 for GABA is not negligible compared to k_{-1}. (If the number of sites for saturable uptake is assumed to be the same for all four substrates, then at 37 °C k_2 for GABA is about 3 times that for L-valine, 4 times that for AIB, and 20 times that for L-lysine.) In principle these explanations can be distinguished in part by noting whether a van't Hoff plot of $\log K_t$ *vs.* $1/T$ is straight (first explanation only) or curved (second, or both first and second explanations); however, the experimental methods do not give sufficiently precise data. Despite the temperature dependence of K_t, $(E_a)_{V_{max}}$ fits the reference pattern, and is evidence that the saturable uptake of GABA, once it has reacted with the carrier, may be similar to the saturable uptake of L-lysine and AIB.

The activation energy for the saturable component of L-valine transport is less than half that for the corresponding component of AIB or L-lysine transport. Its value, 8 kcal/mole, is at the lower limit of the range of values, about 8 to 16 or 20 kcal/mole, for typical enzyme catalyzed reactions (Dixon & Webb, 1964). (The range of values for "typical" mediated transport is not known but presumably is similar.) It is too high for free diffusion, and therefore is evidence that this, like saturable components for the other amino acids, is a mediated process.

Surprisingly, V_{max} for L-valine is essentially independent of temperature. There is no obvious explanation for this result. The corresponding activation energy, -1 kcal/mole, cannot be the true activation energy for a mediated transport process, because mediated transport, like normal catalyzed or uncatalyzed chemical processes proceeding slowly enough at room temperature to permit the use of "classical" methods for measuring reaction rates, must have an appreciable Arrhenius activation energy. This low activation energy can be formally explained by postulating that σ, the density of transport sites, is less by a factor of 2 to 3 at 37 °C (body temperature) than at 25 °C. Intuitively this is highly improbable. An alternate explanation, that regulatory mechanisms reduce the rate of influx much more at 37 °C than at 25 °C, appears unobjectionable. It does raise the question of why the regulation of L-valine uptake is much more temperature dependent than is the regulation of AIB, L-lysine, or GABA uptake.

The Unsaturable Component

Two obvious questions about the nature of the "diffusive component" or "unsaturable component" as it is variously called are: 1) Is it carrier mediated? and 2) What is the rate equation? A number of workers have proposed that the rate-limiting step is not simple diffusion through membranes. In an important paper, Christensen and Liang (1966) argue that the nonsaturable entry of amino acids into ascites cells and rat-intestinal segments must be carrier-mediated because it has a high temperature coefficient; because the rate constant, which they treat as k_D, a diffusion constant (*see below*), depends more on the specific substrate than can reasonably be explained by differences in diffusion rates (e.g., the constants for L- and D-alanine are 3 times that for β-alanine); and because the rate constant depends markedly on the pH of the medium. The same reasoning indicates that the unsaturable component for the uptake of amino acids by brain slices is carrier-mediated, since the activation energies for k_u are appreciable (8 to 41 kcal/mole) and k_u's for the isomers AIB and GABA differ significantly (*see* Table 1).

Two rate equations are commonly used to describe this component:

$$v = k_D(S - S_i) \tag{4}$$

and

$$v = k_u S. \tag{5}$$

The processes they describe are fundamentally different. Eq. (4) predicts no concentrative uptake by this component. It is applicable to nonmediated transport ("simple diffusion"), and to mediated transport where the carrier transports the substrate indifferently in either direction through the membrane and the concentration of substrate is too low to saturate the carrier appreciably [that is, where $k_D = V_{\max}/(K_t + S) \approx V_{\max}/K_t$]. Eq. (5) gives concentrative uptake and requires a carrier. Even if it follows Eq. (5) the carrier may be saturable but require a high substrate concentration to decrease noticeably the apparent first-order rate constant. At substrate concentrations of 100 to 250 mM, k_u for the uptake of AIB by mouse brain slices decreases somewhat (S. R. Cohen, *unpublished*). However, because the medium is no longer an isotonic buffered, balanced saline, with glucose but becomes a hypertonic buffered amino-acid saline with glucose, this decrease may be due to the change in the medium. Matthews (1972) has pointed out that it may be almost impossible to distinguish experimentally between the rate equation for uptake by two saturable components in parallel, that is

$$v = V_{\max_1}/(1 + K_{t_1}/S) + V_{\max_2}/(1 + K_{t_2}/S) \tag{6}$$

and the rate equation for one saturable and one unsaturable component (Eq. 1), if K_{t_2} is very much greater than K_{t_1} and if the maximum experimentally acceptable concentration of the substrate is appreciably less than K_{t_2}. (In such a case $k_u \approx V_{\max_2}/K_{t_2}$.) The distinction between these two rate equations is ignored or blurred in much of the literature. It is often impossible to tell from published data whether the observed component follows diffusion kinetics or the kinetics for first-order uptake. It may be referred to as "diffusion" although first-order kinetics are assumed; or conversely, it may be called "unsaturable" transport although diffusion kinetics are assumed. The Akedo-Christensen plot (1962) is frequently used to evaluate this component of uptake. It was derived assuming the rate law for diffusion, but it also fits first-order kinetics (*see* Appendix). Consequently, it does not distinguish between these two rate equations. The computed parameters $V_{\max}$ and k_u (or k_D) depend however on the assumed kinetics (*see* Appendix) and may be changed by a factor of 2 or 3 in some cases. For example, if diffusion kinetics are assumed for this component of AIB uptake by rat hypothalamus median eminence (Table 2), $V_{\max}$ will be 0.20 instead of 0.063 μmoles/g final wet wt × min; and k_u will be 0.080 instead of 0.025 μmoles/g final wet wt mM-AIB × min.

An unsaturable component or a "diffusive" component has frequently been reported for the influx of amino acids into solid tissues *in vitro* and into suspensions of isolated cells[3]. Typical examples include: brain (Smith, 1967; Laššánová & Brechtlová, 1971; Silverman, Knigge & Peck, 1972; Vahvelainen & Oja, 1972); choroid plexus (Lorenzo & Cutler, 1969; Coben, Cotlier, Beaty & Becker, 1971); diaphragm (Akedo & Christensen, 1962); chick heart (Guidotti, Borghetti, Gaja, Lo Reti & Foà, 1968); kidney (Segal & Crawhall, 1968); iris ciliary body (Coben, Cotlier, Beaty & Becker, 1970); ascites cells (Jacquez *et al.*, 1970; Matthews, 1972); red cells (Eavenson & Christensen, 1967; Gardner & Levy, 1972); embryonic heart cells (Guidotti, Borghetti, Lüneburg & Gazzola, 1971); and *Streptococcus faecalis* (Reid, Utech & Holden, 1970; Utech, Reid & Holden, 1970). A similar component has been observed for other substrates; e.g., the uptake of uridine and adenosine by cultured hepatoma cells (Plagemann, 1970); and glucose by *Pseudomonas aeruginosa* (Eagon & Phibbs, 1971). This component is not always present. Numerous examples of active transport without it are known, including systems with two or more kinetically distinct components.

3 Where the data permit it I am assuming that "diffusive" components and unsaturable components which are *assumed* to obey diffusion kinetics (Eq. (4)) are actually examples of unsaturable uptake described by Eq. (5).

The unsaturable component often provides a significant pathway for influx (Cohen, 1973*a*). Typical values for the fraction of substrate transported by this process are presented in Table 3. Of all the examples in this article (including untabulated values that can be computed from the constants in Table 2), the only exception for central nervous tissue is the uptake of GABA by mouse brain slices at 25 °C; and even here one-fifth of the influx at a concentration of 20 mM is by way of the unsaturable component. The listings for Ehrlich ascites cells illustrate the enormous difference, depending on substrate, in the relative importance of this pathway in a typical preparation. [Values for the other substrates, L-alanine, L-phenylalanine, L-methionine, and glycine, studied by Jacquez *et al.* (1970) lie between these extremes.] The values for the uptake of L-tryptophan by a suspension of ascites cells are higher than the corresponding values for the uptake of AIB by embryonic chick heart or the uptake of GABA by brain slices at 25 °C, showing that the unsaturable component is not an artifact arising from the finite rate of diffusion of substrate into solid-tissue preparations.

Appendix

The Akedo-Christensen Extrapolation

Akedo and Christensen (1962) derived a relation between S_i/S, the ratio of the intracellular concentration of a substrate to its concentration in the medium, and t, the incubation period, by integrating the rate equation over the incubation time. Assuming no initial intracellular concentration and the rate equation

$$v = V_{max}/(1 + K_t/S) + k_D(S - S_i) \tag{7}$$

for saturable uptake plus passive diffusion, they obtained

$$S_i/S = \frac{V_{max}}{k_D}(1 - e^{-k_D t})\left(\frac{1}{S + K_t}\right) + (1 - e^{-k_D t}). \tag{8}^{\star}$$ [their Eq. 4]

At high concentrations this reduces to

$$S_i/S = \frac{V_{max}}{k_D}(1 - e^{-k_D t})\left(\frac{1}{S}\right) + (1 - e^{-k_D t}). \tag{9}^{\star}$$ [their Eq. 2]

Applying the same procedure to Eq. (1), the rate equation for saturable uptake and unsaturable uptake in parallel, gives

$$S_i/S = V_{max}\, t\left(\frac{1}{S + K_t}\right) + k_u t \tag{10}$$

* The notation differs from that used by Akedo and Christensen.

which reduces to

$$S_i/S = V_{\max} t \left(\frac{1}{S}\right) + k_u t \tag{11}$$

at high substrate concentration. Therefore, for either rate equation a plot of S_i/S against $1/(S+K_t)$, or, at high substrate concentrations, against $1/S$ will be linear.

These equations must be modified slightly for brain slices, and other preparations where the true intracellular concentration of substrate cannot be determined, but only the concentration in tissue or in tissue water can be measured. The relation between the intracellular concentration, the tissue concentration, S_t, and the extracellular concentration, S_e, is

$$S_i = S_t(1 + w_e/w_i) - S_e w_e/w_i \tag{12}$$

where w_e and w_i are, respectively, the fraction of tissue that acts as extracellular water or as intracellular water. If we assume that all substrate which enters the functional extracellular spaces, enters by diffusion, then the ratio S_e/S is a function of the preparation and of the incubation time and not of the concentration of the substrate in the medium. If the substrate does not cause tissue swelling or shrinkage, the ratio w_e/w_i is also independent of the substrate concentration. Therefore if Eq. (12) is substituted in Eqs. (8) to (11) their functional form will not be changed. The ratio S_t/S will remain a linear function of $1/(S+K_t)$ or $1/S$, and consequently an Akedo-Christensen plot cannot discriminate between saturable uptake plus passive diffusion, and saturable uptake plus unsaturable uptake. The interpretation of the slope and intercept will depend, however, on the assumed rate equation and on whether the concentration ratio used is S_i/S or S_t/S.

It is of some interest to compare the different forms of the Akedo-Christensen extrapolation equation. Substituting Eq. (12) into Eq. (10) and rearranging gives

$$S_t/S = \left(\frac{w_i}{w_e+w_i}\right)\left\{V_{\max} t \left(\frac{1}{S+K_t}\right) + k_u t\right\} + S_e w_e/S(w_e+w_i). \tag{13}$$

This differs from Eq. (10) by the scale factor $w_i/(w_e+w_i)$ multiplying $V_{\max}$ and k_u, and by the added term $S_e w_e/S(w_e+w_i)$. The scale factor converts $V_{\max}$ and k_u from uptake per unit quantity of intracellular fluid to uptake per unit quantity of tissue fluid or unit weight of tissue (depending on the units of w_e and w_i), and hence justifies the units employed in this paper for $V_{\max}$ and k_u. For sufficiently long incubation times such as used in these

studies, $S_e = S$; the added term then becomes $S^0 = w_e/(w_e + w_i)$. This quantity, the functional extracellular space (Cohen, 1972) of the substrate, is given by the intercept of the linear portion of a graph of uptake *vs.* time (Fig. 1). It can be subtracted from S_t/S if desired. An exactly equivalent result can be found for saturable uptake plus passive diffusion by substituting Eq. (12) into Eq. (8). The resultant equation, when suitably rearranged, is

$$S_t/S = \left(\frac{w_i}{w_e + w_i}\right)\left\{V_{\max} t \left(\frac{1}{S + K_t}\right) + k_D t\right\}(1 - e^{-\tau})/\tau + S_e w_e/S(w_e + w_i) \quad (14)$$

where $\tau = k_D t$ is a dimensionless quantity, the reduced time. The analogous form of Eq. (8) is

$$S_i/S = \left\{V_{\max} t \left(\frac{1}{S + K_t}\right) + k_D t\right\}(1 - e^{-\tau})/\tau. \quad (15)$$

The only difference between Eqs. (15) and (10) or between (14) and (13) (apart from the trivial replacement of k_u by k_D) is the scale factor $(1 - e^{-\tau})/\tau$ multiplying the right-hand side. If $e^{-\tau}$ is expanded in a power series this becomes

$$(1 - e^{-\tau})/\tau = 1 - \tau/2! + \tau^2/3! - \tau^3/4! + \cdots. \quad (16)$$

When the incubation period is short compared to $1/k_D$, that is when τ is small compared to 1, the scale factor will be only slightly less than 1. In this case k_D computed from the intercept assuming passive diffusion (Eq. 7) will be only slightly greater than k_u computed from the intercept assuming unsaturable uptake (Eq. 1); and similarly $V_{\max}$ computed from the slope assuming passive diffusion will be only slightly greater than $V_{\max}$ computed assuming unsaturable uptake. As t becomes larger, the scale factor becomes increasingly smaller; k_D and $V_{\max}$ computed assuming diffusion kinetics become increasingly greater than k_u and $V_{\max}$ computed assuming unsaturable uptake. (K_t does not depend on the assumed kinetics.) For t much greater than $1/k_D$, that is for $\tau \gg 1$, $(1 - e^{-\tau})/\tau \approx 1/\tau$. In this case $k_D \approx k_u \tau$, and $V_{\max\,(\text{diffusion})} \approx V_{\max\,(\text{unsaturable})}\tau$. In principle this provides a way to distinguish between the two rate equations, (1) and (7). Akedo-Christensen graphs are plotted for several incubation times; k_u and k_D (or $V_{\max\,(\text{diffusion})}$ and $V_{\max\,(\text{unsaturable})}$) are both computed from the data. If (1) is the correct rate equation k_u (or $V_{\max\,(\text{unsaturable})}$) will be constant while k_D (or $V_{\max\,(\text{diffusion})}$) will increase without bound; if (7) is correct, k_D (or $V_{\max\,(\text{diffusion})}$) will be constant while k_u (or $V_{\max\,(\text{unsaturable})}$) will approach zero. Whether this procedure is practical depends on several factors including the quality of the data, and the importance of efflux or regulation at higher incubation times (*see* Fig. 1).

I wish to thank Dr. Amos Neidle at the Institute for the amino acid analyses and for helpful discussions about this article.

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